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## EFFECT OF BLOOD TRANSFUSIONS ON IMMUNE FUNCTION. VIII. EFFECT ON MACROPHAGE RESPONSE TO TUMOR CHALLENGE

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Blood transfusions have previously been demonstrated in Wistar-Furth rat models to impair resistance to pulmonary metastases from a colonic tumor, but not the growth of the primary tumor. Utilizing the same tumor cell line we evaluated the effect of transfusion on resistance to peritoneal spread of the tumor and on macrophage response to the transfusion. Transfusions were found to have no significant effect on survival in animals with peritoneal carcinomatosis, nor on the host response to the tumor. Transfusion failed to alter macrophage function or metabolism as measured by macrophage cytotoxicity against YAC-1 cells, by macrophage production of adenosine deaminase, by glucose metabolism, or by macrophage ATP levels. In summary, transfusions failed to alter the peritoneal resistance to tumor spread or the activity of the predominant resident peritoneal leukocyte.

**KEY WORDS:** Transfusion, immune function, macrophage, tumor.

### INTRODUCTION

It has previously been demonstrated in a number of retrospective studies that there is an adverse correlation between perioperative blood transfusions and long-term survival in colon cancer patients.<sup>1-4</sup> However, this correlation has not been noted universally.<sup>5</sup> Part of the difficulty with such retrospective studies is the possibility that these patients with more advanced or aggressive tumors (lead time or length bias) received a greater proportion of the transfusions. To eliminate the potential for such

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The care of all animals was in accordance with the guidelines set forth by the Animal Welfare Act and other Federal statutes and regulations relating to animals and studies involving animals and with the *Guide for the Care and Use of Laboratory Animals*, National Institutes of Health Publication 86-23.

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bias, animal studies have been utilized. These studies have also yielded contradictory results, depending on the species and strain of animal utilized, the type of tumor, and the method of transfusion.<sup>6</sup>

We have previously demonstrated, utilizing a Wistar-Furth colon cancer model, that allogeneic transfusions do not increase the rate of primary tumor growth, at least with this tumor line. When the same tumor was utilized in a pulmonary metastases model, we found an enhanced rate of growth of pulmonary metastases and a decreased long-term survival rate in allogeneically transfused rats as compared to a control group which received lactated Ringer's. The purpose of our current study was to determine if this alteration in host response to pulmonary metastases is representative of a universal response to metastases in transfused rats or if it is unique to pulmonary metastases.

## MATERIALS AND METHODS

### *Animals*

Adult male Wistar-Furth rats weighing approximately 250 g were used in these studies. Adult male A'Sogaloff Cancer Institute (ACI) rats were used as blood donors. All animals were housed in individual stainless steel, hanging cages and allowed food and water *ad libitum* throughout the study. The animals were observed for 1 week prior to entry into the study to exclude the possibility of any preexisting diseases.

### *Transfusion Protocol*

Blood was obtained from the donor rats by vena cava puncture and mixed at a 4:1 volumes ratio with CPDA-1 anticoagulant. Animals in the control group received 3 ml of lactated Ringer's solution intravenously and those in the transfusion group were given 1 ml of ACI rat blood intravenously. The increased volume of lactated Ringer's was chosen since it was believed that this would closely approximate the intravascular volume changes achieved in the rats receiving 1 ml of whole ACI rat blood.

### *Tumor Protocol*

Two tumor models were used to evaluate the transfusion effect on tumor growth. In the first model, 45 rats (control = 28, transfusion = 27) received  $1 \times 10^6$  tumor cells suspended in 1 ml of complete RPMI injected intraperitoneally through a 23-ga needle. The tumor cell suspension was prepared as previously described.<sup>6</sup> Briefly, viable tumor was obtained by excision of a rapidly growing syngeneic 1,2-dimethylhydrazine-induced colon carcinoma. The tumor was mechanically disaggregated by first slicing the tumor into approximately 1-mm<sup>3</sup> pieces and vigorously shaking the suspension in complete RPMI-1640 media with penicillin, streptomycin, and 10% fetal calf serum. The cells were washed 3X in the same media. An aliquot of this suspension was stained with trypan blue and the number of viable tumor cells determined. The cells were centrifuged and resuspended in sufficient RPMI to achieve a final concentration of  $1 \times 10^6$  viable cells/ml. A 1-ml aliquot of this suspension was injected intraperitoneally through a 23-ga needle into the Wistar-Furth rats immediately following the administration of either lactated Ringer's or blood. Animals were

followed to death and mean survival times were determined. All animals were necropsied to confirm that death was due to tumor growth. Eighty-five days after implantation of the tumor cell suspension, all rats remaining alive appeared healthy and had no evidence of viable tumor growing intraperitoneally by physical examination. These rats were sacrificed by a lethal sodium pentobarbital injection and were necropsied. None of these rats were found to have any macroscopic evidence of tumor present. For the purpose of calculating mean survival time, all of these animals were given a survival time of 85 days, and for calculating survival rates, these rats were considered to be permanent survivors.

Leukocyte infiltration of the tumors present in the animals described above was assayed as previously described.<sup>7</sup> Briefly, wedge biopsies of the periphery of the tumors were performed at the time of necropsy. The biopsies were fixed in formalin, sectioned, and stained with hematoxylin-eosin. Cellular infiltrates were quantified by enumeration of cells at the tumor periphery in 15 hpf with an image analysis system (Optomax<sup>R</sup>, Hollis, NH).

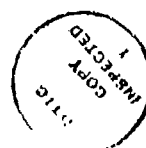
For the second tumor model, a similar tumor cell suspension was prepared. Twenty-two Wistar-Furth rats (control = 10, transfusion = 12) were anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg). A midline celiotomy was performed and 1 ml of the tumor cell suspension was injected into the retroperitoneum. The incisions were closed in multiple layers and the animals were allowed to awaken in their cages. These animals were followed to mortality and mean survival times determined.

To evaluate the effect of transfusion on macrophage cytotoxic response to tumor, elicited peritoneal macrophages were studied. Twenty Wistar-Furth rats were administered either 1 ml of ACI rat blood ( $n = 10$ ) or 3 ml of lactated Ringer's ( $n = 10$ ). Three days following administration of the transfusion or lactated Ringer's, 4 ml of brain-heart infusate was administered intraperitoneally through a 23-ga needle. Four days later, the animals were sacrificed by decapitation. A midline celiotomy was performed and the peritoneal cavity lavaged with 20 ml of Hank's balanced salt solution (HBSS) without calcium or magnesium and with 0.25 mM EDTA. The resulting suspensions were hypotonically lysed of any contaminating red cells and washed 3X in standard HBSS. They were resuspended in RPMI-1640 with 10% fetal calf serum. The resulting macrophage suspensions were assayed for macrophage purity and for macrophage cytotoxic activity against YAC-1 cells.

Macrophage purity was tested for by both adherence to flat-bottomed cell well plates (96 wells) (Corning Glass Works, Corning, NY) and by nonspecific esterase staining. Macrophage adherence was tested for by placing  $5 \times 10^5$  cells in 0.1 ml of media per well. The plates were cultured for 1 hour at 37°C in 5% CO<sub>2</sub>. Nonadherent cells were then removed and the remaining cells were counted. Ninety percent of the cells from both the transfused and control rats were found to be adherent. Nonspecific esterase staining was assayed using a standard esterase staining kit (Kit #90-A1, Sigma Technical Bulletin #90). Ninety-eight percent of the cells from both the transfused and control rats were found to be positive for esterase staining.

Macrophage cytotoxicity against YAC-1 cells was assayed as follows. Briefly,  $1 \times 10^6$  YAC-1 cells/ml RPMI-1640 with 10% fetal calf serum and containing 5  $\mu$ Ci/ml of <sup>125</sup>I-labelled 5-iodo-2'-deoxyuridine (Dupont Corp, NEN Research Products, Boston, MA) plus  $10^{-6}$  M 5-fluorouridine (Sigma Chemical Corporation) were cultured at 37°C in 7.5% CO<sub>2</sub> for 4 hours. The cultures were then washed three times to remove all nonincorporated radioactivity.  $5 \times 10^4$  of the labelled YAC-1 cells in

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0.1 ml RPMI-1640 with 2.5% fetal calf serum were added to each well in the cell well plates which contained  $5 \times 10^5$  macrophages in 0.1 ml RPMI with 2.5% fetal calf serum. Additional wells containing only target cells were utilized for measuring spontaneous release. Total counts were measured by lysis of target cells utilizing detergent. All wells were cultured at 37°C for 24 hours. Supernatants from each well were harvested and assayed for radioactivity present. Specific release for each well was calculated as follows:

$$\frac{\text{cpm released in test well} - \text{cpm released in spontaneous control sample}}{\text{cpm in total lysis well} - \text{cpm in spontaneous release sample}}$$

Data are presented as percent of mean of the value for macrophages obtained from the control group.

The effect of transfusion on macrophage metabolism was assayed by measuring the amount of adenosine deaminase produced by cultured macrophages, the intracellular levels of adenosine triphosphate (ATP) and finally glucose metabolism by the macrophages. Briefly, 20 Wistar-Furth rats were transfused ( $n = 10$ ) or given lactated Ringer's ( $n = 10$ ) and had peritoneal macrophages elicited as described above 3 days after transfusion. Four days after BHI injection, the macrophages were harvested and purified as described above.  $1 \times 10^7$  of the cells were suspended per milliliter of complete RPMI media and 1-ml aliquots of the suspension were cultured in standard flat-bottomed polystyrene culture plates with concanavalin A stimulation for 6 hours at 37°C. The plates were then frozen at -70°C and thawed at room temperature three times. Aliquots of the lysed suspensions were analyzed for glucose, adenosine deaminase, and adenosine triphosphate levels. Glucose content of the RPMI media prior to macrophage addition was also assayed.

Glucose was determined on a Beckman Synchron CX3 System (Beckman Instruments, Inc., Brea, CA). The Synchron CX3 glucose chemistry determines glucose by means of the oxygen-rate method employing a Beckman oxygen electrode.

Adenosine deaminase activity was assayed using a colorimetric method described by Giusti.<sup>8</sup> Briefly, 0.05-ml aliquots of the thawed macrophage lysate were added to 1 ml of buffered adenosine solution (21 mM adenosine, 50 mM phosphate buffer, pH 6.5). After incubation for 60 min at 37°C, 3.0 ml of phenol/nitroprusside solution (10<sup>6</sup> mM phenol, 0.17 mM sodium nitroprusside) and 3.0 ml of alkaline hypochlorite solution (11 mM NaOCl, 125 mM NaOH) were added. After incubation for 30 min at 37°C, absorbance at 628 nm (E) was measured. Volume activity was determined as follows:

$$(\text{E sample} - \text{E sample blank}) \div (\text{E standard} - \text{E reagent blank})$$

Adenosine triphosphate levels were measured by a coupled enzymatic reaction.<sup>9</sup> Briefly, adenosine triphosphate was measured using the coupled enzymatic reaction:  $\text{ATP} + 3\text{-phosphoglycerate} \rightarrow \text{ADP} + 1,3\text{-diphosphoglycerate}$  [1]  $1,3\text{-diphosphoglycerate} + \text{NADH} \rightarrow \text{glyceraldehyde 3-phosphate} + \text{NAD} + \text{phosphate}$  [2]. Reaction [1] was catalyzed by phosphoglycerate kinase and reaction [2] was catalyzed by glyceraldehyde phosphate dehydrogenase. The change in absorbance at 340 nm that results when NADH is oxidized to NAD was measured on a Gilford 240 spectrophotometer (Gilford Instruments, Gerdin, OH). Enzymes and reagents were obtained from Sigma Chemical (St. Louis, MO).

### Statistical Analysis

All data are expressed as mean  $\pm$  SEM. Comparisons among groups were made using Fischer's exact, generalized Savage (Mantel-Cox), and ANOVA.

## RESULTS

The survival curves for the animals challenged with intraperitoneal tumor cell suspensions are shown in Figure 1. There were 19 deaths and 9 survivors in the control group. In the transfused group, there were 12 deaths and 15 survivors. This difference was not statistically significant ( $P = 0.106$ ). The mean survival time for the control group was  $60.71 \pm 3.80$  days and for the transfusion group,  $70.81 \pm 3.58$  days. This difference was also not statistically significant ( $P = 0.0629$ ). Necropsy of each of the deaths revealed a generalized peritoneal carcinomatosis.

Analysis of leukocyte infiltration of the tumors revealed  $123.0 \pm 38.4$  cells/hpf in the tumors obtained from the control group and  $143.9 \pm 41.9$  cells/hpf in the tumors obtained from the transfused group. This difference was not statistically significant ( $P = 0.196$ ).

The survival curves for the animals challenged with retroperitoneal tumor cell suspensions are shown in Figure 2. The mean survival time for the control group was  $34.50 \pm 2.15$  days and for the transfusion group,  $33.67 \pm 2.63$  days. This difference was not statistically significant ( $P = 0.883$ ).

The macrophages obtained from the control group had  $100.0 \pm 11.8\%$  of the predicted lysis of the YAC-1 cells. The macrophages obtained from the transfused rat had  $121.1 \pm 16.3\%$  of the predicted lysis of the YAC-1 cells. This difference was not statistically significant ( $P = 0.305$ ).

The glucose level in the lysed suspensions of macrophages obtained from the control rats was  $188.4 \pm 0.5$  mg/dl. The glucose level in the lysed suspensions of macrophages obtained from the transfused rats was  $189.7 \pm 1.1$  mg/dl. This difference was not statistically significant ( $P = 0.387$ ). The glucose level from five samples of RPMI media prior to macrophage addition was  $192.0 \pm 0.7$  mg/dl, indicating a similar slow rate of glucose metabolism in both groups.

The adenosine deaminase level in the lysed suspensions of macrophages obtained

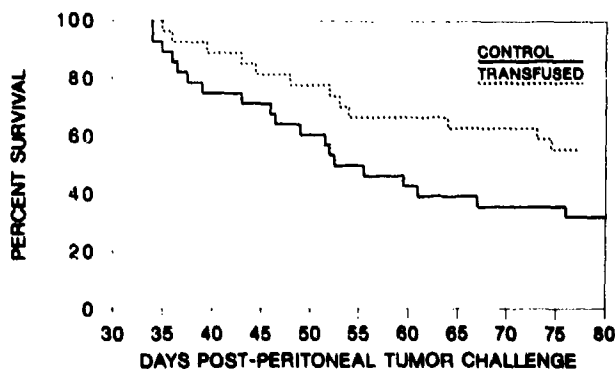


FIGURE 1 Survival curves for control and transfused groups challenged with  $1 \times 10^6$  tumor cells intraperitoneally.

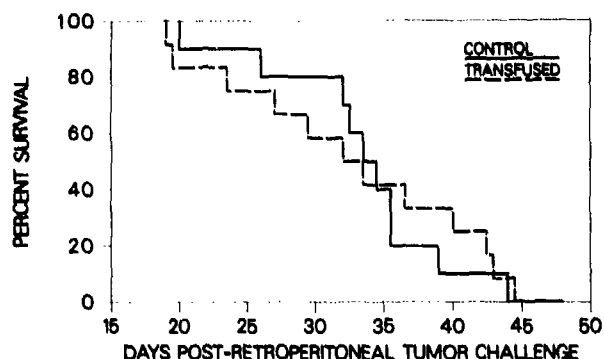


FIGURE 2 Survival curves for control and transfused groups challenged with  $1 \times 10^6$  tumor cells retroperitoneally.

from the control rats was  $3.33 \pm 1.78$  U/ml and with supernatants from the lysed suspensions of macrophages obtained from transfused rats,  $1.06 \pm 0.19$  U/ml. This difference was not significant ( $P = 0.147$ ). The greater mean and the large standard error of the mean for the control group was due to a single sample which had a value far in excess of all other samples in both groups.

The adenosine triphosphate level of the lysed suspensions of macrophages obtained from the control rats was  $9.57 \pm 1.26 \mu\text{g}/1 \times 10^7$  cells and for the lysed suspensions of macrophages obtained from transfused rats,  $10.66 \pm 1.56 \mu\text{g}/1 \times 10^7$  cells. This difference was not significant ( $P = 0.638$ ).

## DISCUSSION

Appropriate surgical intervention is normally able to control the primary site of common solid malignant neoplasms. It is, rather, the distant metastases which eventually lead to a fatal outcome. Such metastases can take place by three methods, migration through coelomic cavities, spread through lymphatic vessels, and spread through blood vessels.

Tumor spread by these methods does not always lead to a successful metastasis. For such tumor spread to eventually become a metastasis, the tumor cells, or group of tumor cells, must implant in a distant site and escape control by the host's immune system. The patient's immune system can generate a complex immune response to both the primary tumor and metastatic sites. Among the more important components of this response are helper/inducer T lymphocytes, cytotoxic T lymphocytes, natural killer cells, and cytotoxic macrophages.

We have previously reported, utilizing the same tumor cell line in Wistar-Furth rats, that blood transfusions can alter certain components of the host's response to this tumor when the tumor is presented in a particular manner.<sup>6</sup> In that study allogeneic blood transfusions were demonstrated to have no significant effect on the host's response to the primary tumor site with this cell line. This finding was confirmed in our current study in that, when all the tumor cells were injected into a single site in the host's retroperitoneum, transfusions exerted no effect on survival. Our current study also confirmed our previous finding that transfusions do not appear to

alter the host's response to tumor growths once they become established since as in our earlier study,<sup>6</sup> transfusions did not alter the leukocyte infiltration of established tumor growths, in this case, the peritoneal carcinomatosis growths.

Our previous study with this tumor cell line did demonstrate that transfusions increased the rate of growth of pulmonary metastases and decreased long-term survival in rats bearing pulmonary metastases when the tumor cells were given intravenously and thus allowed to implant throughout the lungs as multiple single-cell emboli. Since natural killer cells are particularly important in a successful host response to blood borne tumor cells, we also evaluated the effect of allogeneic transfusions on natural killer cell function. It was found that such transfusions significantly impaired natural killer cell cytotoxicity against YAC-1 cells at both 1 and 2 weeks following the transfusions. This finding would appear to indicate that a selective impairment of a single component of the immune system may alter the clinical response of the host at least to a particular method of tumor spread.

Our current study was therefore designed to determine if the alteration in host response to pulmonary metastases following transfusion was representative of a universal response to metastases or merely of a particular immune component and a selective organ response to metastases. Although the allogeneic blood source, the tumor host, and the tumor cell line were identical to our previous study, the host's response to the metastatic tumor cell challenge (peritoneal) and the response of the particular immune cell studied (macrophage) were different. There was noted to be no effect of the transfusion on macrophage cytotoxicity against YAC-1 cells. The transfusions also failed to alter the metabolic activity of the macrophages, as measured by glucose metabolism, adenosine deaminase levels, or adenosine triphosphate levels. These findings would appear to indicate that there was no significant effect of the transfusions on the ability of the macrophages to be stimulated to a metabolically excited and immunologically enhanced state by either concanavalin A mitogen or tumor cell targets. This could explain the lack of a transfusion effect on the peritoneal metastatic carcinoma model used in our study, since such macrophages are the initial immune response cell in the host's peritoneum.

The lack of a transfusion effect on the macrophage's metabolic parameters measured in our current model is somewhat surprising in view of the previous demonstration of a transfusion effect on macrophage arachidonic acid metabolism.<sup>10</sup> That study found that transfusions increased the rate of metabolism of arachidonic acid by the macrophage's cyclooxygenase enzyme system. This discrepancy may reflect that the alterations in macrophage activity following transfusion relate primarily to alterations in macrophage regulation of other components of the immune system,<sup>11</sup> rather than alterations in the macrophage's response to invading pathogens be they tumor or microorganisms.<sup>12</sup>

Our current study thus provides further confirmation of the selectivity of the transfusion effect. Just as the transfusion effect has been shown to alter resistance to gram-negative infections<sup>13,14</sup> but not to gram-positive infections,<sup>15,16</sup> so it also impairs resistance to primary tumor growth of certain tumor cell lines<sup>17,18</sup> but not to the primary growth of other tumor lines.<sup>19</sup> Of even greater interest is the finding that its ability to alter resistance to gram-negative infections and tumor cell metastases is dependent upon whether the challenge is intravenous or intraperitoneal.<sup>6,20</sup>

Finally, our current study indicates that the transfusion-induced alterations in immune function may provide a model for investigating the effect of various components of host immune function on the response to tumor challenge. In the future,

such an understanding might assist in the formulation of immunostimulatory protocols for the treatment of oncology patients.

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